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1 ***Yersinia enterocolitica* specific infection by bacteriophages TG1 and ϕ R1-RT is**
2 **dependent on temperature regulated expression of the phage host receptor**

3 **OmpF**

4

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24

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29

30 **ABSTRACT**

31 Bacteriophages present huge potential both as a resource for developing novel tools
32 for bacterial diagnostics and for use in phage therapy. This is also valid for
33 bacteriophages specific for *Yersinia enterocolitica*. To increase our knowledge on *Y.*
34 *enterocolitica* –specific phages we characterized two novel yersiniophages. The
35 genomes of the bacteriophages vB_YenM_TG1 (TG1) and vB_YenM_φR1-RT (φR1-
36 RT), isolated from pig manure in Canada and from sewage in Finland, consist of
37 linear double-stranded DNA of 162,101 and 168,809 bp respectively. Their genomes
38 encode 262 putative coding sequences and 4 tRNAs genes, and share 91% overall
39 nucleotide identity. Based on phylogenetic analyses of their whole genome sequences
40 and large terminase subunit protein sequences, a genus named *Tg1virus* within the
41 family *Myoviridae* is proposed with TG1 and φR1-RT as member species. These
42 bacteriophages exhibit a host range restricted to *Y. enterocolitica*, and display lytic
43 activity against the epidemiologically significant serotypes O:3, O:5,27, and O:9 at
44 and below 25°C. Adsorption analyses of LPS and OmpF mutants demonstrate that
45 these phages use both the LPS inner core heptosyl residues and the outer membrane
46 protein OmpF as phage receptors. Based on RNA-sequencing and quantitative
47 proteomics we also demonstrate the temperature dependent infection is due to strong
48 repression of OmpF at 37°C. In addition, φR1-RT was shown to be able to enter into a
49 pseudolysogenic state. All together, this work provides further insight into phage-host
50 cell interactions by highlighting the importance of understanding underlying factors
51 which may affect the abundance of phage host receptors on the cell surface.

52

53 **IMPORTANCE**

54 Only a small number of bacteriophages infecting *Y. enterocolitica*, the predominant
55 causative agent of yersiniosis, have been previously described. Here, two newly
56 isolated *Y. enterocolitica* phages were studied in detail with the aim of elucidating the
57 host cell receptors required for infection. Our research further expands the repertoire
58 of phages available for consideration as potential antimicrobial agents or as diagnostic
59 tools for this important bacterial pathogen.

60 **INTRODUCTION**

61 *Yersinia enterocolitica*, a facultative anaerobic, Gram-negative, non-sporulating, short
62 bacillus isolated frequently from soil, water, animals, and foods, is an important
63 zoonotic pathogen leading to human and animal enteric infection (1). The main
64 animal reservoir for *Y. enterocolitica* is pigs, and pork derived products are thought to
65 be the main source of human infections in addition to drinking of contaminated water
66 and blood-transfusions (1, 2). Symptoms of yersiniosis may include diarrhea, terminal
67 ileitis, mesenteric lymphadenitis, and septicemia (3). Among the species within the
68 genus *Yersinia*, *Y. enterocolitica* is highly heterogeneous and is grouped into six
69 phylogroups (4). The widely used bioserotype groups form the basis of the
70 phylogroups such that phylogroup 1 contains the biotype 1A strains, phylogroup 2 the
71 highly pathogenic biotype 1B strains, phylogroup 3 the bioserotype 4/O:3 strains,
72 phylogroup 4 bioserotype 3/O:9 strains, phylogroup 5 bioserotype 2/O:5,27 strains
73 and phylogroup 6 the serotype O:2,3 strains rarely isolated from hares (4–7). *Y.*
74 *enterocolitica* is also represented by over 60 serotypes that are determined by the
75 variability of O-antigens present in the outer cell membrane (8, 9). The predominant
76 pathogenic strains associated with yersiniosis belong to bioserotypes 1B/O:8,
77 2/O:5,27, 2/O:9, 3/O:3, and 4/O:3, with the last being the most common in Europe,
78 Japan, Canada, and the United States (1, 2). From 2010–2012, 98% of all reported
79 yersiniosis infections worldwide were acquired in Europe, and most (97%) were
80 caused by *Y. enterocolitica*, with the remainder caused by *Y. pseudotuberculosis* (10).
81 In 2015, the most commonly reported *Y. enterocolitica* serotype in the European
82 Union was O:3 (89%), followed by serotypes O:9 (7%), O:5,27 (2%) and O:8 (2%)
83 (10).

84 Although several bacteriophages infecting *Y. enterocolitica* have been described, few
85 have been studied in detail providing reliable information on morphology, host range,
86 and or receptor specificity. To date, bacteriophages ϕ YeO3-12(11–13) and
87 vB_YenP_AP5 (14) with specificity for *Y. enterocolitica* O:3, phage PY54 exhibiting
88 a host range restricted to *Y. enterocolitica* O:5 and O:5,27 (15), *Yersinia* phage ϕ R1-
89 37 with a broad host range within the species *Y. enterocolitica* (16, 17) and *Yersinia*
90 phage PY-100 (18) exhibiting a broader host range restricted to the genus *Yersinia*,
91 have been described. These bacteriophages use different parts of the *Y. enterocolitica*
92 lipopolysaccharide (LPS) as receptors (19). Analysis of the host-range combined with
93 genetic and structural data have shown that the receptor for ϕ R1-37 is the *Y.*
94 *enterocolitica* O:3 LPS outer core (OC) hexasaccharide (16). The host receptor for
95 phages ϕ YeO3-12 and vB_YenP_AP5 has been determined to be the LPS O-antigen
96 of serotype O:3 consisting of the sugar 6-deoxy-L-altropyranose (12, 14, 20). Given
97 the interest in bacteriophages because of their potential use as therapeutic, diagnostic,
98 and bio-control agents, the aim of this study was to characterize two newly isolated
99 bacteriophages that are active against several epidemiologically significant *Y.*
100 *enterocolitica* serotypes. In this study, the genome characterization, morphology, host
101 range, host cell receptor specificity, and taxonomic position of the myovirus phages
102 vB_YenM_TG1 (hereafter TG1) and vB_YenM_ ϕ R1-RT (hereafter ϕ R1-RT) are
103 described.

104

105 **MATERIALS AND METHODS**

106 **Bacterial strains, phage isolation, and growth conditions.** Bacterial strains,
107 bacteriophages and plasmids are listed in **Table 1**. Bacteriophage ϕ R1-RT was
108 isolated from the incoming sewage of the Turku (Finland) city sewage treatment

109 plant, as described for other viruses (19) whereas bacteriophage TG1 was isolated
110 from pig manure collected from a rural farm in Ontario, Canada as described
111 previously for the isolation of *Y. enterocolitica* phages for phagetyping (21). For
112 DNA extraction and morphological studies, ϕ R1-RT was propagated on *Y.*
113 *enterocolitica* strain YeO3-R1 (22) and TG1 on *Y. enterocolitica* strain YeO3-c (23).

114

115 **Electron Microscopy.** The preparation of the phage particles for transmission
116 electron microscopy (TEM) was done as described (17, 24). Details are presented in
117 Supplementary Materials and Methods.

118

119 **Host Range.** The lytic activity of ϕ R1-RT and TG1 was tested on 109 and 160 strains
120 (**Table S1**), respectively, belonging to 13 *Yersinia* species, as determined by standard
121 spot tests (24). Briefly, 10 μ l from a phage suspension containing approximately 10^8
122 PFU were spotted in the middle of a lawn of bacteria incubating for 18-24 h. Each
123 strain was tested three times at 25°C and at 37 °C. Bacterial strains were considered
124 sensitive to the phage if the degree of lysis was observed as a complete clearing,
125 clearing throughout but with a faint hazy background, substantial turbidity throughout
126 the cleared zone, or a few individual plaques (24). Bacterial strains were considered
127 resistant if there was no effect of the phage on bacterial growth.

128

129 **Genome sequencing and assembly.** Details of the determination of the genomic
130 sequences of phages ϕ R1-RT and TG1 as well as the draft genomes of *Y.*
131 *enterocolitica* strains YeO3- ϕ R1-RT-R2, -R7 and -R9 are presented in
132 Supplementary Materials and Methods.

133

134 **Bioinformatics.** Detailed description of the bioinformatics tools used is given in
135 Supplementary Materials and Methods.

136

137 **Complementation of the *Y. enterocolitica* O:3 OmpF mutant.** The full ORF of
138 *ompF* gene plus the upstream promoter region of YeO3-c was cloned as a 2 kb PCR
139 fragment that was amplified with Phusion DNA polymerase using primer pair OmpC-
140 F2 and OmpC-R2 (**Table S2**) into plasmids pTM100 and pSW25T to obtain plasmids
141 pTM100_OmpF and pSW25T_OmpF, respectively (**Table 1**). Briefly, the PCR
142 fragments were digested with MfeI and ligated with EcoRI digested, SAP-treated
143 pTM100 or pSW25T. The constructed plasmids were mobilized to the OmpF mutant
144 strain YeO3-c-R1-Cat17 by diparental conjugation as described earlier (25).

145

146 **Phage adsorption assay.** To identify the phage cell host receptors, a variety of *Y.*
147 *enterocolitica* O:3 mutants (**Table 1**) were utilized in phage adsorption experiments.
148 Approximately 5×10^3 PFU of phage ϕ R1-RT or phage TG1 in 100 μ l was mixed with
149 a 400- μ l sample of bacteria ($A_{600} \sim 1.2$). The suspension was incubated at RT for 5
150 min and centrifuged at 16,000 *g* for 3 min, and the phage titer remaining in the
151 supernatant, *i.e.*, the residual PFU percentage, was determined. LB was used as a non-
152 adsorbing control in each assay, and the phage titer in the control supernatant was set
153 to 100%. Each assay was performed in duplicate and repeated at least three times.

154

155 **Total RNA extraction and RNA sequencing.** Detailed description of the methods is
156 presented in Supplementary information Materials and Methods. The RNA sequence
157 data has been deposited to Gene Expression Omnibus (accession number GSE66516).

158

159 **Quantitative proteomics.** Detailed description of the methods is presented in
160 Supplementary information Materials and Methods.

161

162 **Transduction assay.** *Y. enterocolitica* O:3 strain YeO3-*hfq*::Km with *hfq* gene
163 knocked-out with a kanamycin resistance cassette (**Table 1**) was used as a donor and
164 transducing particles were produced by infecting this strain with phage ϕ R1-RT using
165 the soft agar overlay method. Following overnight incubation, phages were eluted
166 from the soft agar using SM buffer. The transducing lysates were centrifuged and
167 treated with chloroform to prevent contamination with the donor strain. The titer of
168 the obtained transducing stock was 6.62×10^9 PFU/mL. The *Y. enterocolitica* strain
169 6471/76 was used as the recipient. For the transduction of the recipient strain, 10 one
170 mL aliquots of log-phase bacterial cultures containing 10^9 CFU/mL cells were mixed
171 with 100 μ l of 10^{-2} diluted transducing phage stock resulting at MOI of 0.006. After
172 15 min the bacterial cells were centrifuged and washed with LB and centrifuging
173 them down removed the unabsorbed phages. The final cell pellet was resuspended in
174 100 μ l LB, and the cells were allowed to recover during 30 min incubation with
175 vigorous shaking. Subsequently, the bacterial cultures were plated on urea agar plates
176 (0.1% peptone, 0.1% glucose, 0.5% NaCl, 0.2% KH_2PO_4 , 0.00012% phenol red, 2%
177 urea, 1.5% agar) supplemented with kanamycin (200 μ g/mL) and incubated for 48h.
178 The kanamycin resistant and urease negative colonies were considered as transduced.
179 The transducing stock was also plated to ensure no contamination with donor strain.

180

181 **Growth curves.** Overnight bacterial cultures were diluted 1:10 in fresh LB medium
182 and 180 μ l aliquots were distributed into honeycomb plate wells (Growth Curves Ab
183 Ltd) where they were mixed with 20 μ l aliquots of different ϕ R1-RT phage stock

184 dilutions ($10^0 - 10^{-4}$). A negative control was obtained by mixing 20 μ l of phage stock
185 with 180 μ l of medium, whereas positive control consisted of 180 μ l of bacterial
186 culture and 20 μ l of medium. The growth experiments were carried out at 4°C, 10°C,
187 16°C, 22°C, and 37°C using the Bioscreen C incubator (Growth Curves Ab Ltd) with
188 continuous shaking. The OD₆₀₀ of the cultures was measured at selected time
189 intervals. The averages were calculated from values obtained for the bacteria grown in
190 5 parallel wells.

191

192 **Phage resistant mutant isolation.** A culture of wild type *Y. enterocolitica* strain
193 6471/76 was used to flood LB agar plates (LA). After the excess fluid was removed
194 the plates were allowed to dry before two 100 μ l aliquots of the ϕ R1-RT stock were
195 pipetted on the lawn of cells. The plates were incubated at 22°C and inspected daily
196 for phage resistant colonies growing within the lysis zones. After three days several
197 colonies appeared and among them three confirmed phage resistant derivatives were
198 isolated. The strains were named YeO3- ϕ R1-RT-R2, YeO3- ϕ R1-RT-R7, and YeO3-
199 ϕ R1-RT-R9.

200

201 **CatMu-library screening.** The *CatMu*-transposon insertion library in *Y.*
202 *enterocolitica* strain YeO3-R1 has been described elsewhere (26, 27). In the present
203 work, a library representing 16,000 independent insertion mutants was screened. The
204 library was grown in LA supplemented with 100 μ g/ml chloramphenicol (LA-CIm)
205 until OD₆₀₀ = ~0.5. Phage ϕ R1-RT was added to 1 mL of the library culture at MOI
206 ~10, fresh LB added to 5 mL and the culture was incubated at 22°C for 2h during
207 which time all phage-sensitive bacteria were expected to be infected and lysed. The
208 surviving bacteria were pelleted by centrifugation, washed twice with 1 mL LB to

209 remove remaining phages and after resuspending into 100 μ l of LB plated on four
210 LA-Clm plates that were incubated at 22°C. The Clm^R colonies were re-streaked on
211 LA-Clm plates for further study.

212

213 **Arbitrary PCR.** Detailed description of the method is presented in Supplementary
214 information Materials and Methods.

215

216 **Cloning, expression and purification of the phage long tail fiber host receptor**

217 **binding protein.** The phage TG1 distal long tail fiber (LTF) protein Gp37 was co-

218 expressed with phage encoded chaperones Gp57A and Gp38 to synthesize the native

219 form of the putative receptor binding protein (RBP) as described previously for the

220 LTF of phage T4 (28). The Gp37 encoding gene was first cloned into the multiple

221 cloning site (MCS) 1 of pCDF Duet-1 (conferring streptomycin resistance), producing

222 pCDF Duet-1 Gp37. Then, the Gp38 encoding gene was cloned into the MCS 2 of

223 pCDF Duet-1 Gp37, yielding pCDF Duet-1 Gp37-Gp38. Plasmid pET21a(+)

224 conferring ampicillin resistance was used to clone the chaperone Gp57A encoding

225 gene yielding plasmid pET21a(+) Gp57A. The plasmid constructs carry under the

226 control of promoter T7, high level inducible gene expression with a His₆ fusion tag at

227 the N-terminus for purification by chelating affinity chromatography (**Fig. S1**). The

228 genes encoding Gp38 and Gp57A however, were expressed without a purification tag.

229 PCR, restriction analysis, and DNA sequencing were used to verify the structure of

230 the plasmids. For expression, *E. coli* BL21 StarTM (DE3) PLYS cells (Invitrogen)

231 were transformed with pCDF-Duet-1 Gp37 or pCDF-Duet-1 Gp37-Gp38 and the

232 same plasmids were also co-transformed with pET21a(+) Gp57A. Plasmid bearing *E.*

233 *coli* were grown aerobically at 37°C to an OD₆₀₀ = ~0.6 with shaking at 200 rpm in

234 250 mL of 2xYT media (16 g/L tryptone, 10 g/L yeast extract, 5.0 g/L NaCl, 0.22 μ m
235 filter sterilized, pH 6.5-7.5) supplemented with 50 μ g/mL of ampicillin and or 50
236 μ g/mL streptomycin as required. Protein expression was induced by the addition of 1
237 mM isopropyl-d-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich, USA) incubating
238 for 24h at 30°C with shaking at 200 rpm. Cells were harvested by centrifugation at
239 10,000 g for 15 min at 4°C and the pellets were resuspended in 25 mL of buffer A (50
240 mM sodium phosphate, 300 mM NaCl, 10mM imidazole, pH 8.0) supplemented with
241 a protease inhibitor cocktail (Roche). Cells were disrupted by 10 rounds of 15 s of
242 sonication using a Virsonic Digital 475 ultrasonicator (VirTis, NY, USA) alternating
243 with incubation on ice. Insoluble debris was removed by centrifugation at 18,000 g
244 for 30 min at 4°C and the soluble fraction was filtered through a 0.22 μ m pore size
245 filter (EMD Millipore, USA). The protein was purified by immobilized metal ion
246 affinity chromatography using a nickel-nitrilotriacetic acid (Ni-NTA) agarose column
247 (Novex, Invitrogen) according to the manufacturer's protocol. Captured proteins were
248 eluted from the column using buffer B (50 mM sodium phosphate, 300 mM NaCl,
249 500mM imidazole, pH 8.0) and concentrated using Amicon-Pro centrifuge filters
250 (Millipore) with a 10,000 Da molecular mass exclusion limit incorporating three
251 washes with 10 mM Tris-HCl of pH 8.5. Protein concentration was estimated by
252 measuring sample absorbance at 280 and 260 nm using a Nanodrop 2000 UV-vis
253 Spectrophotometer (Thermo Scientific, USA) and Qubit® Protein Assay Kit using a
254 Qubit® 1.0 fluorometer (Life Technologies) as per the manufacturer instructions.
255 Protein analysis was performed by Sodium dodecyl sulfate-polyacrylamide gel
256 electrophoresis (SDS-PAGE) (29) using Mini-Protean®TGX Stain-Free Precast Gels
257 (Bio-Rad Laboratories, USA) and Coomassie blue staining. Precision Plus Protein™
258 Unstained Standard (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used as a

259 size marker for the molecular analysis of proteins. Analysis of protein bands and
260 molecular weight (MW) estimates was performed using a Molecular Imager® Gel
261 Doc™ XR+ System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and Quantity
262 One® software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Accurate MW
263 determinations and Peptide mass fingerprinting analysis was performed via mass
264 spectrometry (MS) at the Mass Spectrometry Facility, Advanced Analysis Centre of
265 the University of Guelph (Ontario, Canada).

266

267 **Cell decoration with bacteriophage host recognition binding proteins.** Confocal
268 laser immunofluorescent microscopy was used to visualize the binding of the phage
269 TG1 LTF protein Gp37 to *Y. enterocolitica* following methodology described by
270 others (30). *Yersinia* strains grown in TSB at 25°C or 37°C for 24 hours were
271 resuspended in wash buffer (50 mM Tris-HCl, pH 7.5) and 10 µl were spotted onto
272 clean glass slides. After air-drying, the cells were fixed in a solution of 5 %
273 gluteraldehyde for 10 min. and blocked with blocking buffer (5% BSA in 50 mM
274 Tris-HCl buffer, pH 7.5) for 10 min. The slides were then incubated for 1 hour in a
275 solution containing of 10 µg/mL of phage TG1 Gp37 (prepared in blocking buffer)
276 followed by washing three times for 5 minutes in wash buffer. The slides were then
277 incubated for 1 hour in anti-His₆ tag (HIS.H8) mouse monoclonal antibody solution
278 prepared in blocking buffer (1:1000 dilution) and washed three times for 5 minutes
279 with wash buffer. In a dark room, the slides were then incubated for 1 hour in goat
280 anti-mouse IgG DyLight 488 polyclonal antibody solution (1:500) prepared in
281 blocking buffer and washed three times for 5 minutes with wash buffer. The slides
282 were air dried prior to analysis. Cells were imaged using an upright Leica DM 6000B
283 confocal laser microscope connected to a Leica TCS SP5 system. Images were

284 collected digitally using Leica LAS AF Imaging Software and processed using
285 ImageJ (31). To verify the specificity of the fluorescent signal, control samples were
286 immunolabelled as above, with the omission of incubation with the primary antibody.
287 All antibodies were acquired from Pierce Scientific, USA.

288

289 **Genome sequences.** The complete genome sequences of *Yersinia* phage
290 vB_YenM_TG1 and vB_YenM_φR1-RT were deposited in the NCBI nucleotide
291 database (GenBank) under the accession numbers KP202158 and HE956709,
292 respectively. The RNA sequence data has been deposited to Gene Expression
293 Omnibus (Acc. no GSE66516).

294

295 **RESULTS**

296 **Phage Morphology.** Phages φR1-RT and TG1 were negatively stained and examined
297 by TEM. Both phages exhibit a prolate head with apparent icosahedral symmetry and
298 a tubular contractile and rigid tail showing transverse striations (**Fig. 1**). The average
299 dimension for the φR1-RT head is 82 ± 4 nm short edge-to-edge, 101 ± 5 nm vertex-
300 to-vertex and the tail including the baseplate is on average 130 ± 7 nm long. The
301 average dimension for the TG1 head is 91 ± 2 nm short edge-to-edge, 115 ± 6 nm
302 vertex-to-vertex and the tail including the baseplate is on average 129 ± 1 nm long.
303 Collectively, these morphological features indicate that these phages belong to the
304 *Myoviridae* family.

305

306 **Host specificity.** The host range of phages TG1 and φR1-RT were determined by
307 testing their lytic activity on 160 and 109 strains, respectively, belonging to thirteen
308 *Yersinia* species, revealing virulence for *Y. enterocolitica* strains of serotypes O:1,

309 O:2, O:3, O:5, O:6, O:5,27, O:7,8, O:9 and some strains of serotype O:6,30 and
310 O:6,31 while strains from other *Y. enterocolitica* serotypes and species within the
311 genus *Yersinia* were resistant to phage infection (**Table 2**). TG1 and ϕ R1-RT lysed
312 their host when grown at 25°C but not at 37°C. Additionally, TG1 was unable to
313 infect strains belonging to other 20 other genera (**Table S3**) demonstrating the
314 phages' host range is restricted to *Y. enterocolitica*.

315

316 **General features of the phage genomes.** The genome of phage ϕ R1-RT is 168,809
317 bp long with a GC content of 34.5%. The genome encodes 262 ORFs, of which 217
318 genes are encoded on the reverse strand (as displayed on the genetic map) and 45
319 genes on the forward strand (**Fig. S2**), with sizes ranging from 117 bp (product of 38
320 amino acids) to 3,738 bp (product of 1245 amino acids). The genome of TG1 is
321 smaller than that of ϕ R1-RT at 162,101 bp in length but with a similarly low GC
322 content of 34.6%. TG1 also encodes 262 ORFs of which 223 genes are transcribed on
323 the reverse strand (as displayed on the genetic map) and thirty-nine genes on the
324 forward strand (**Fig. S3**), with sizes ranging from 114 bp (product of 37 amino acids)
325 to 3,099 bp (product of 1032 amino acids). The GC content of these phages is
326 significantly lower than that associated with the host with a GC content ranging from
327 47.1 ± 0.2 (32) to 48.5 ± 1.5 % (33). The genomes encode additionally four identical
328 tRNA genes (Gly_{GGA}, Trp_{TGG}, Arg_{AGA}, Met_{ATG}) identified using tRNAScan (34) and
329 ARAGORN (35). Constitutively low GC phage genomes are often supplemented with
330 tRNA genes that, once expressed, enhance translation efficiency when infecting high
331 GC content hosts (36). At the DNA level the TG1 genome shows 98% identity with a
332 query coverage of 93%, for an overall DNA sequence identity of 91% with ϕ R1-RT.
333 All ORFs were screened using the BLASTP and PSI-BLAST algorithms (37, 38).

334 Based on protein homology, putative functions could be assigned to 121 (46%) gene
335 products of phage TG1 and 115 gene products (44 %) of phage ϕ R1-RT. Most of the
336 identified homologs are conserved among T4-like phages and are either structural, or
337 involved in DNA replication, recombination, repair, or packaging. Thus, the phage T4
338 gene nomenclature was used to name these genes (**Table S4**).

339

340 **DNA replication, recombination, and repair.** Numerous genes were identified
341 within the phage ϕ R1-RT and TG1 genomes that play a direct role in DNA
342 replication, recombination, and repair. Among the genes directly involved in DNA
343 replication are a DNA polymerase, a DNA ligase (Gp30), and three proteins with
344 helicase activity. The closest homologs to the phage TG1 and ϕ R1-RT polymerases
345 are found in *Edwardsiella* phage PEi20 [BAQ22701.1] and *Enterobacteria* phage
346 RB69 [NP_861746.1]; all members of the *Myoviridae*. Among the helicases, Gp41
347 (or Dda) and UvsW homologs are involved in the reorganization of stalled DNA
348 replication forks (39). Other putative proteins identified include homologs to the
349 DNA polymerase sliding clamp loader complex Gp44/Gp62, sliding clamp accessory
350 protein Gp45, single-stranded DNA binding protein Gp32, DNA helicase loader
351 Gp59, and Gp61. In phage T4, the latter is a primase that interacts with helicase Gp41
352 to form a helicase-primase complex (or primosome). The primosome together with
353 the DNA helicase loader Gp59, unwinds the DNA template and primes DNA
354 synthesis on the discontinuous strand. Among the proteins involved in recombination
355 are type II topoisomerases Gp60 and Gp52, the recombination-related endonuclease
356 pair Gp46/Gp47, the Rec-A like recombination protein UvsX, and a single stranded
357 DNA binding protein, UvsY (40). Lastly, among the proteins involved in repair, a
358 DenV homolog and several RNA ligases were identified. DenV is an N-glycosylase

359 UV repair enzyme that excises pyrimidine dimers; the major UV-lesions of DNA,
360 while RNA ligases seal breaks in RNA and may also counteract host defense of
361 cleavage of specific tRNA molecules (41).

362

363 **Nucleotide metabolism.** Class I ribonucleotide reductases are responsible for the
364 inter-conversion of ribo- to deoxyribonucleotides and are represented by NrdA-B or
365 NrdE-F which require oxygen for activity, class II containing NrdJ, and the oxygen
366 sensitive class III represented by NrdG-H (42). In TG1 and ϕ R1-RT, genes coding for
367 the aerobic ribonucleotide reductase complex subunits NrdA, NrdB, and NrdH were
368 identified. Additionally, NrdC genes were also located. Other genes identified that are
369 involved in nucleotide metabolism include: thymidylate synthase (*Td*), thymidine
370 kinase (*Tk*), dNMP kinase, dCMP deaminase (*Cd*), dihydrofolate reductase (*Fdr*),
371 dCTPase-dUTPase, and the exo-deoxyribonuclease *DexA* and endo-
372 deoxyribonuclease *DenA*. A combination of at least some of these genes is required to
373 supplement the intracellular pool of nucleotides for phage DNA and RNA synthesis
374 (41).

375

376 **Transcription.** Based on the genome maps presented (**Fig. S2 and S3**), phage TG1
377 and ϕ R1-RT present a similar gene arrangement. A search for promoters based on
378 sequence similarity to the host consensus *s70* promoter TTGACA(N15-18)TATAAT
379 with a 2 bp mismatch, identified 22 probable host promoters in the phage TG1
380 genome and 24 probable host promoters in the ϕ R1-RT genome which probably
381 function in early transcription (**Tables S5 and S6**). Additionally, 15 of the putative
382 host promoters are located in the same relative genomic positions within each phage
383 genome. The genomic layout however, makes it clear that there must be additional

384 promoters functioning to direct the transition from host to viral metabolism. A search
385 for phage-specific promoters using PHIRE (43) and by analysis of sequences of 100
386 bp in length upstream of each ORF and submitting them to MEME (44), did not yield
387 additional promoters that could be annotated with confidence. A search for putative
388 rho-independent transcription terminators using ARNold (45, 46) yielded 21 putative
389 terminators in the phage TG1 genome (**Table S7**) and 24 in the phage ϕ R1-RT
390 genome (**Table S8**). Nevertheless, the presence phage T4 homologs involved in the
391 transcription of late genes: RegA, Gp33, and the sigma factor for late transcription
392 Gp55, suggest that the mechanism for controlling late transcription is similarly
393 complex (41). Likewise, the presence of repressor and translational regulatory protein
394 homologs involved in middle and late transcription including: RegB, DsbA, Alc,
395 MotA, and AsiA, lend further support to this suggestion.

396

397 **Morphogenesis.** The putative structural proteins of TG1 and ϕ R1-RT are
398 homologous to existing phage proteins of the T4 supergroup of viruses (**Table S4**).
399 Among the putative phage structural genes, the phage head is likely composed of the
400 major capsid protein Gp23 and the phage capsid vertex protein Gp24. The prohead
401 precursor and scaffolding proteins Gp68, and Gp67 as well as internal head proteins
402 ipIII and ipII were also identified. Lastly the head portal vertex protein Gp20 that is
403 connected to the neck and through which DNA enters during packaging and exits
404 during infection was also identified. The whiskers and neck are composed of fibritin
405 (*wac*) and the head completion proteins Gp13 and Gp14. The tail proteins include the
406 tail sheath terminator Gp3, the tail completion protein Gp15, the tail sheath subunit
407 Gp18, and the tail tube subunit Gp19. Proteins that form the baseplate wedge subunits
408 and tail pins that then go on to associate with the central hub to form the viral

409 baseplate include: Gp5, Gp6, Gp7, Gp8, Gp9, Gp10, Gp11, Gp25, Gp27, Gp28, and
410 Gp53. Among these, Gp5 (*ORF150*) contains a predicted bacteriophage T4-like
411 lysozyme domain (cd00735) or Phage lysozyme domain (pfam00959), which aids
412 penetration through the peptidoglycan layer during the initial infection process. In
413 phage T4, Gp8 and Gp9 connect the long tail fibers of the virus to the baseplate and
414 trigger tail contraction after viral attachment to a host cell, while Gp11 connects the
415 short tail fiber protein Gp12 (*ORF159*) to the baseplate (47). The baseplate wedge
416 subunit Gp25, forms a structural component of the outer wedge of the baseplate that
417 has lysozyme activity, evident by the presence of conserved Gene 25-like lysozyme
418 domain (pfam04965). Based on homology and gene synteny the proteins forming the
419 long tail fibers in TG1 and ϕ R1-RT are composed of the tail fiber proximal subunit
420 Gp34, the tail fiber connector or hinge protein Gp35, the proximal tail fiber protein
421 Gp36, and the distal tail fiber protein Gp37 (47). A variety of chaperones or assembly
422 catalysts involved in morphogenesis were also discovered. Head formation
423 chaperones include the capsid vertex assembly chaperone, the prohead assembly
424 proteins Gp21 and Gp22, as well as the head assembly chaperone protein Gp31.
425 Chaperones involved in tail formation include the baseplate hub assembly proteins
426 Gp26 and Gp51. Chaperones for tail fiber assembly include gp57A, gp57B, and
427 Gp38.

428

429 **Host cell recognition elements.** In phage T4, phage tail associated receptor-binding
430 proteins (RBPs) Gp37 and Gp12 are necessary for host cell recognition, attachment,
431 and initiation of infection. In the phage TG1 and ϕ R1-RT genomes *ORF250* codes for
432 a putative RBP protein of 609 amino acid residues and 503 amino acid residues in
433 length, respectively, sharing 60% overall sequence identity. These proteins also share

434 40% sequence identity to the distal long tail fiber RBP of *Cronobacter* phage
435 vB_CsaM_GAP161 [YP_006986537.1] and are homologs to the long tail fiber RBP
436 Gp37 of phage T4 [AJC64544.1]. An alignment of these two proteins reveals a high
437 degree of conservation at the N-terminus associated with the proximal tail fiber, as
438 well as at the C-terminus associated with host recognition (**Fig. S4**). More
439 specifically, the C-terminal 63 amino acids present a 95% sequence identity.
440 Similarly, in the phage TG1 and ϕ R1-RT genomes, *ORF159* codes for the short tail
441 fiber (STF) protein Gp12, both of 446 amino acid residues in length and which are
442 almost identical to each other, sharing 95% overall sequence identity (**Fig. S5**). These
443 proteins are homologous to the STF protein Gp12 of phage T4 [NP_049770.1].

444

445 **DNA Packaging.** In phage TG1, *ORF164* and *ORF165/ORF167* genes code for the
446 small (TerS) and large (TerL) DNA packaging subunits respectively of a phage
447 terminase protein complex (or holoterminase) that initiates, drives, and terminates
448 translocation of phage DNA into proheads (48). The homologous genes in phage
449 ϕ R1-RT are represented by *ORF165* (TerS) and *ORF166/ORF168* (TerL). Usually,
450 *terS* and *terL* are arranged side by side but in phage TG1 and ϕ R1-RT two ORFs
451 homologous with *terL* are found. *ORF165* in TG1 and *ORF166* in ϕ R1-RT show
452 sequence similarity to the N-terminus of the phage T4 TerL. Likewise *ORF167* in
453 TG1 and *ORF168* in ϕ R1-RT show sequence similarity to the C-terminus of phage T4
454 TerL. BLASTX analysis (37, 38) reveals the *terL* gene in both phages is interrupted
455 by a transposase (PHA02552). Additionally, during packaging, the DNA ends are also
456 protected against host RecBCD nuclease action by Gp2, the DNA end protector
457 protein (49); identified in phage TG1 and in ϕ R1-RT as the product of *ORF146*.

458 **Homing Endonucleases.** Homing endonuclease genes (HEGs) are not genuine phage
459 DNA, but rather belong to intron associated selfish DNA elements (50) and are
460 commonly found interspersed throughout *Myoviridae* genomes (41). Among the
461 HEGs identified in phage TG1, *ORF148* and *ORF232* exhibit similarity to shortened
462 helix-turn-helix (HN-H) endonucleases, and *ORF9*, *ORF43*, and *ORF66* to GIY-YIG
463 group I intron endonucleases. BLASTX analysis (37, 38) reveals Gp47
464 (recombination-related endonuclease II) is divided by *ORF66* which contains the
465 HEG. Likewise, the gene coding for UvsX is intersected by *ORF43*, which contains
466 the HEG. *ORF232* also divides the *NrdA* gene. In phage ϕ R1-RT five HEGs are also
467 found throughout the genome of which only *ORF148* is homologous to the helix-turn-
468 helix (HN-H) endonuclease that is also located in phage TG1 between Gp4 and Gp53.
469 *ORF20*, *ORF51*, *ORF163*, and *ORF234* exhibit similarity to GIY-YIG group I intron
470 endonucleases, none of which interrupt or intersect other ϕ R1-RT genes.

471

472 **Lysis.** The final stage of the phage lytic cycle involves the degradation of the bacterial
473 cell wall and release of progeny phages induced by the effect a pore producing
474 protein, the holin, and a peptidoglycan degrading enzyme, the endolysin (51). In TG1,
475 *ORF127* and *ORF122* in ϕ R1-RT each encode an obvious endolysin containing a
476 bacteriophage T4-like lysozyme protein domain (pfam00959) and phage-related
477 muramidase (COG3772). Access of the endolysin to the cell wall occurs through the
478 presence of the holin. Holins are small phage encoded proteins characterized by the
479 presence of TMDs, accumulating in the cytoplasmic membrane during infection until
480 suddenly at a specific time, trigger to form lethal lesions resulting in destruction of the
481 cell wall (51, 52). A search for the TG1 and ϕ R1-RT holins revealed the putative
482 product of their respective *ORF253* gene contains a predicted t-holin domain

483 (pfam11031) with 70% identity to the phage holin of *Enterobacteria* phage CC31
484 [YP_004010117.1]. The protein sequences are predicted to contain a single TMD
485 spanning aa interval 30-49, as well as a large C-terminal periplasmic domain spanning
486 the aa residue from position 50 to the end terminal amino acid at position 218; a
487 characteristic bitopic topology found in the holin proteins of T4-like phages (53)
488 Moreover, as in phage T4 the putative holin gene is separated from the endolysin
489 gene. An additional search for *Rz/RzI* genes coding for transmembrane spanins
490 involved in the disruption of the outer membrane of the host was also conducted
491 based on gene arrangement and membrane localization signals (54). The search
492 revealed two candidate genes, *ORF225* and *ORF224* in phage TG1 and *ORF227* and
493 *ORF226* in phage ϕ R1-RT, homologous to phage T4 pseT.3 (*Rz*) and pseT.2 (*RzI*),
494 respectively. As in phage T4, the *Rz/RzI* genes are adjacent to each other, arranged
495 with overlapping stop and start codons, and additionally no part of the *RzI* sequence
496 is embedded within the *Rz* coding region (54). In TG1 and ϕ R1-RT, *Rz* possesses a
497 single amino-terminal TMD, and *RzI* encodes an outer membrane lipoprotein based
498 on the presence of a signal peptidase II (SPII) cleavage site located between amino
499 acid residues 16 and 17 as predicted by LipoP (54, 55). Lastly, the presence of phage
500 T4 homologs to *rI* lysis inhibition regulator membrane protein and *rIII* lysis inhibitor
501 accessory protein in TG1 and ϕ R1-RT suggest the potential for lysis inhibition (LIN)
502 following superinfection (56, 57).

503

504 **Phylogeny of TG1.** It is interesting to note that very similar bacteriophages with an
505 overall DNA sequence identity of 91% were isolated from such different locations
506 and sources, as phages TG1 and ϕ R1-RT were isolated in Canada from pig manure,
507 and in Finland from raw sewage, respectively. Moreover, less than 34% overall DNA

508 similarity exists with their closest neighbours within the *Myoviridae* (**Table S9**). The
509 relatedness of these two phages was further explored using progressiveMauve (**Fig. 2**)
510 (58, 59); CoreGenes (60, 61) which the Bacterial and Archaeal Virus Subcommittee
511 of the International Committee on Taxonomy of Viruses (ICTV) has extensively used
512 to compare the proteomes of viruses; and by phylogenetic analysis of their whole
513 genome sequences (**Fig. S6**) and their large terminase subunit protein sequences (**Fig.**
514 **3**). It is evident from phylogenetic analyses that TG1 and ϕ R1-RT form a distinct
515 taxonomic clade among their closest neighbours. Based on these observations and
516 using a 95% DNA sequence identity as the criterion for demarcation for a species, a
517 new genus named *Tg1virus* with phages TG1 and ϕ R1-RT as member species was
518 proposed to the ICTV (approved in 2016 and pending ratification).

519

520 **Growth curves.** In order to study the efficiency of phage infection at different
521 temperatures bacterial growth after phage infection with ϕ R1-RT was measured. Host
522 bacterial strain was grown at selected temperatures with addition of different phage
523 stock dilutions. Bacterial growth was followed for 3 d at 4°C, 2d at 10°C and 16°C
524 and 1 d at 22°C and 37°C. Lysis of the bacterial cultures was observed at 4°C, 10°C,
525 16°C and 22°C, whereas at 37°C the bacteria were not significantly affected even
526 with the highest initial phage concentrations (**Fig. 4**, panels A). The onset time of
527 lysis depended on the temperature and initial phage titer. At 4°C the bacterial culture
528 started to lyse after 56-60 h, at 10°C already at 16 h with highest phage titer and at
529 24-28 h with the lowest phage titer. The corresponding times for 16°C and 22°C were
530 6 and 12 h. While the lysis at 10°C and 16°C was complete, at 22°C strong regrowth
531 after the initial lysis took place. At 4°C the 3 d incubation time was not long enough
532 to follow the lysis to completion. Under all tested conditions negative (medium only)

533 controls showed no increase in the absorbance, whereas the positive (bacteria only)
534 controls presented the normal bacterial growth pattern.

535

536 **Transduction.** To study the transducing potential of ϕ R1-RT, transduction of the
537 Km^R and urease-negative phenotype of strain YeO3-*hfq*:: Km to the Km^S and urease-
538 positive wild type strain 64741/76 was assayed. Repression of urease activity is one
539 of the phenotypes of the *hfq* mutant (62) and could be used to confirm the
540 transduction of the *hfq*:: Km allele. The transduction assays were performed in 10
541 parallel tubes using a MOI of 0.006. A total of 6.6×10^8 PFU from the transducing
542 lysate resulted in a total of 3 Km^R urease-negative colonies that were confirmed by
543 PCR. From this the calculated transduction frequency in the experiment was 4.5×10^{-7}
544 transductants per PFU.

545

546 **Identification of the phage receptors – pseudolysogeny.** As the LPS and protein
547 profiles of the phage resistant mutants YeO3- ϕ R1-RT-2, YeO3- ϕ R1-RT-7, and
548 YeO3- ϕ R1-RT-9 did not differ from those of the wild type bacteria (data not shown)
549 the genomic DNA of the mutant and the wild type strains were sequenced. The *de*
550 *novo* assembly results showed that the total scaffold sizes of the assembled genomes
551 of the three mutants were ~165-173 kb larger than that of the wild type parental strain
552 (**Table S10**). This suggested that the mutants carried extra DNA and the size matched
553 very close to the size of phage ϕ R1-RT genome (168,809 bp). This immediately
554 raised the possibility that the phage had lysogenized the host and would reside as a
555 prophage. In all three draft genomes the phage genome sequence was indeed
556 identified and in all it formed the scaffold 4.1 with almost identical sizes (**Table S10**).
557 Significantly, in all three cases the scaffold sequences were 100% identical to phage

558 ϕ R1-RT sequence without any flanking host sequences, suggesting that the phage
559 genome resided in these bacteria as an autonomous replicating unit in a state known
560 as pseudolysogeny. Such state has been described for T4-like phages (63).

561

562 **Identification of the phage receptors – transposon insertion library screening.** As
563 selection of spontaneous phage resistant mutants seemed to favor pseudolysogeny we
564 decided to use a different approach. A *CatMu*-transposon library of strain YeO3-R1
565 (26) was exposed to the ϕ R1-RT for 2 hr and the surviving phage-resistant mutants were
566 grown on LA-CIm plates. The recovered colonies were tested to be true ϕ R1-RT
567 resistant mutants. In order to exclude pseudolysogens, the clones were screened with
568 ϕ R1-RT specific PCR, and negative ones were further analysed by *CatMu*-specific
569 arbitrary PCR to identify the *CatMu* insertion site (26). For four of the candidates the
570 transposon insertion site was identified as gene *Y11_04441* of the *Y. enterocolitica*
571 O:3 strain Y11 genome (NC_017564.1). In strain Y11 genome the gene was
572 annotated to encode for the outer membrane porin OmpC, however, in all other *Y.*
573 *enterocolitica* genomic sequences as OmpF, therefore we opted to use OmpF. To
574 confirm that OmpF is the ϕ R1-RT receptor, one of the mutants YeO3-R1-Cat17 was
575 complemented with the wild type *ompF* gene either *in trans* with plasmid
576 pTM100_*OmpF* or *in cis* by suicide plasmid pSW25T_*OmpF*. Both of these
577 approaches resulted in regaining the phage sensitivity thus confirming that OmpF
578 serves as ϕ R1-RT receptor.

579

580 **The LPS inner core heptose region functions as a receptor.** Adsorption
581 experiments were carried out to study the ability of ϕ R1-RT and TG1 to interact with
582 *Y. enterocolitica* O:3 derivatives differing mainly in their LPS composition (**Fig. 5**). A

583 short 5 min adsorption time was used as it produced highest resolution between the
584 strains. A general observation was that TG1 adsorbed faster than ϕ R1-RT. Both
585 phages showed negligible adsorption to YeO3-c-R1-Cat17, the *ompF* mutant strain
586 and adsorbed well to both *ompF*-complemented strains. Both phages showed reduced
587 but clear adsorption to the pseudolysogen, indicating changes in abundance or
588 exposure of the phage receptor(s). Finally, the adsorption to the inner core mutants
589 decreased with the truncation of the core oligosaccharide suggesting that the inner
590 core heptoses are part of the secondary receptor (**Fig. 5**).

591

592 **Temperature-dependence of *ompF* expression.** We then wondered whether the
593 temperature-dependent sensitivity of *Y. enterocolitica* O:3 could be due to *ompF*
594 regulation. The expression of *ompF* under different growth temperatures was analysed
595 from RNA-sequencing and quantitative proteomics (LC-MS/MS) data. The
596 transcriptomic data showed an inverse correlation between the expression of *ompF*
597 and the temperature of incubation (**Fig. 4**, panels B). Consistently, the quantitative
598 proteomics demonstrated much higher abundance of the OmpF protein in the 22°C
599 sample when compared to the 37°C sample, where the abundance barely exceeded the
600 threshold of identification (**Fig. 4**, panels B).

601

602 **In vitro expression of the long tail fiber protein Gp37 of phage TG1.** In this study,
603 co-expression with the phage encoded chaperones Gp38 (required for
604 oligomerization) and Gp57A, which is also thought to participate in assembly (64, 65)
605 was utilized in an attempt to synthesize the native form of distal long tail fiber protein
606 of phage TG1 as previously described for the production of Gp37 from phage T4 (28).
607 SDS-PAGE demonstrated that an oligomer of approximately 210 kDa was obtained

608 when Gp37 was co-expressed with Gp38 in a bicistronic plasmid (pCDF-Duet-1
609 Gp37-Gp38) or when this same plasmid was co-expressed with Gp57A (**Fig. S7**,
610 lanes 3 and 5). Under reduced conditions Gp37 appears as a monomer of
611 approximately 70 kDa in size (**Fig. S7**, lanes 4 and 6). This estimate is close to the
612 predicted molecular mass of the recombinant phage TG1 Gp37 determined via MS at
613 approximately 68.050 kDa. Peptide mass fingerprinting confirmed the identity of the
614 protein (**Fig. S8**). Based on the protein expression results obtained, it appears that in
615 phage TG1 only the Gp38 chaperone is essential and the general chaperone Gp57A is
616 not required for *in-vitro* protein folding of Gp37 as has been reported for phage T4
617 (28). The formation of higher molecular weight oligomers of phage TG1 Gp37 is
618 consistent with previous reports that describe RBPs of phages present as homotrimers
619 in solution migrating in the SDS-PAGE with a mobility that corresponds to that of
620 oligomeric forms (28, 66–68).

621

622 **Confirmation of host binding specificity.** Host binding specificity was then tested
623 through immunolabeling of bacterial cells with phage TG1 LTF protein Gp37
624 followed by detection with anti-His₆ antibodies and DyLight 488 conjugated
625 secondary antibodies. Consistent with the temperature dependent infection of phage
626 TG1, the application of the LTF protein Gp37 to *Y. enterocolitica* cells showed
627 decoration of the surface of *Y. enterocolitica* O:3, O:5,27, and O:9 cells when these
628 were grown at 25°C but not at 37°C (**Fig. 6**). Notably, binding was more apparent
629 near the apex of the cells which is also reported to occur in other phages such as λ ,
630 T4, T7, KVP40 and ϕ A1122, preferentially infecting cells at the poles (69).

631

632 **DISCUSSION**

633 Among bacteriophages, the C-terminus of RBPs involved in ligand interactions
634 usually exhibits considerable sequence divergence, thus providing diversity in host
635 specificity. In the case of ϕ R1-RT and TG1, the high sequence identity at the C-
636 terminus of their long tail fiber and short tail fiber proteins may account for the
637 striking similarity in virulence of these two phages for *Y. enterocolitica*. Notably,
638 phage ϕ R1-RT shows virulence to strains of the same serotypes as phage TG1. Based
639 on adsorption experiments, the outer membrane protein OmpF and the inner core
640 heptosyl residues of the LPS serve as phage receptors for phage TG1 and ϕ R1-RT. It
641 is worth noting however, that the *E. coli* strain DH10B/pTM100_OmpF was not
642 sensitive to ϕ R1-RT. We reasoned that this could be due to poor expression of *Y.*
643 *enterocolitica* OmpF in *E. coli* or more likely that the LPS inner core, known to be
644 used by T4-like phages as the secondary receptor (76, 77) was not compatible. The
645 inner core structures of *E. coli* and *Y. enterocolitica* differ substantially potentially
646 explaining this result.

647

648 Multiple lines of evidence suggest OmpF is the primary host range determinant for
649 these two bacteriophages. First, a multiple alignment of OmpF amino acid sequences
650 of *Y. enterocolitica* (from a BLASTP search of sequence databases using the O:3
651 OmpF sequence as query) suggest the restricted host range of these phages among *Y.*
652 *enterocolitica* serotypes could be due to OmpF. The alignment provided a distribution
653 of conserved amino acid residues and the presence of regions with high and low
654 homologies, which coincide with eight transmembrane domains and eight “external”
655 loops, respectively of the topology of the OmpF porin from *E. coli* (70, 71). The
656 search and alignment of the sequences (**Fig. S9**) revealed that the OmpF sequences of
657 the ϕ R1-RT sensitive serotypes are 100% identical. The most dramatic differences

658 between the serotypes map to loop 4. In the alignment most close to the O:3 sequence
659 is the serotype O:7,8,19 OmpF that is 96% identical to O:3 and may still be sensitive
660 to ϕ R1-RT; in it the loop 4 sequence differs least, while in others differences are
661 bigger and also accumulate in other loops, mainly in loops 5, 6, and 7 (**Fig. S9**). The
662 porin loops are plausible binding sites for bacteriophages as demonstrated by the
663 interaction of *E. coli* OmpF and K20 phages which bind to the L5, L6, and L7
664 external loops (72–74). Thus it is likely that the loop 4 sequence is targeted by the
665 ϕ R1-RT or TG1 receptor binding proteins, however, experimental evidence is
666 necessary to confirm this. Secondly, RNA-sequencing and quantitative proteomics
667 data, the analysis of growth curves of *Y. enterocolitica* infected with ϕ R1-RT at
668 various temperatures (4°C to 37°C), as well as phage host range analysis results
669 conducted at 25°C and 37°C clearly indicate that the failure of ϕ R1-RT and TG1 to
670 infect *Y. enterocolitica* O:3 at 37°C is due to the strong repression of the *ompF* gene.
671 The temperature dependent expression of OmpF results also agree with a previous
672 study, where two-dimensional gel electrophoresis of whole-cell proteins of *Y.*
673 *enterocolitica* cultured at 25°C and 37°C suggested that OmpF is downregulated
674 when the bacteria were cultured at 37°C (75). Consistent with this observation, the
675 application of immunolabelled phage TG1 receptor binding protein Gp37 to *Y.*
676 *enterocolitica* cells showed decoration of the surface of *Y. enterocolitica* O:3, O:5,27,
677 and O:9 cells when these were cultured at 25°C but not at 37°C. The decoration of the
678 cell surface agrees with a high level expression of this major outer membrane protein
679 class depending on the bacterial species and the environmental conditions, which can
680 reach about 10^4 – 10^6 copies per cell (72). It is reasonable to suggest then that the
681 phage TG1 distal long tail fiber protein Gp37 (and by extension, its homolog in ϕ R1-
682 RT) is specifically involved in binding to OmpF while presumably, the short tail fiber

683 protein Gp12 binds to the inner core of LPS, as is reported to occur in other T even
684 phages as a secondary receptor (76, 77).

685

686 The *in vitro* temperature dependent infection of these two highly related phages,
687 questions their potential use as biocontrol or therapeutic agents as has been suggested
688 for the temperate *Yersinia* phage PY100 (18, 78). On the other hand, it is not known
689 whether the *ompF* gene is expressed *in vivo* justifying further studies towards finding
690 that out. However, due to their marked specificity for the epidemiological relevant *Y.*
691 *enterocolitica* serotypes O:3, O:5,27, and O:9, these phages may prove useful for
692 diagnostic purposes. In addition, the successful synthesis of the long tail fiber of
693 phage TG1 opens up the possibility of its use as a probe as well as for the production
694 of suitable amounts of protein for X-ray crystallography to elucidate its atomic
695 structure or co-crystallization with its receptor OmpF to shed light on specific host
696 cell receptor-virus interactions.

697

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701

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707

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Table 1. Bacterial strains, plasmids and bacteriophages

| Strain | Comments | Reference |
|-------------------------------------|------------------------------------------------------------------------|----------------------------------------------|
| <i>Y. enterocolitica</i> | | |
| 6471/76 (YeO3) | Serotype O:3, wild type. Human stool isolate | (23) |
| 6471/76-c (YeO3-c) | Virulence-plasmid cured derivative of YeO3 | (23) |
| YeO3- ϕ R1-RT-R2 | ϕ R1-RT resistant spontaneous derivative of YeO3 | This work |
| YeO3- ϕ R1-RT-R7 | ϕ R1-RT resistant spontaneous derivative of YeO3 | This work |
| YeO3- ϕ R1-RT-R9 | ϕ R1-RT resistant spontaneous derivative of YeO3 | This work |
| YeO3-R1 | (=YeO3-c-R1) Spontaneous rough strain | (22) |
| YeO3- <i>hfq</i> ::Km | <i>Hfq</i> ::Km-GenBlock, Km ^R . Urease-negative | (Leskinen et al., submitted for publication) |
| YeO3-R1-Cat17 | <i>ompF</i> :: <i>Cat-Mu</i> derivative of YeO3-R1 | This work |
| YeO3-R1-Cat17::pSW25T_OmpF | <i>Cis</i> complemented <i>ompF</i> :: <i>Cat-Mu</i> strain | This work |
| YeO3-R1-M164 | <i>waaF</i> :: <i>Cat-Mu</i> . derivative of YeO3-R1. Clm ^R | (79) |
| YeO3-R1-M196 | <i>galU</i> :: <i>Cat-Mu</i> derivative of YeO3-R1. Clm ^R | (79) |
| YeO3-R1-M205 | <i>hldE</i> :: <i>Cat-Mu</i> derivative of YeO3-R1. Clm ^R | (79) |
| YeO3-c-OC | | (25) |
| YeO3-c-OCR | | (25) |
| K14 | Serotype O:9 | |
| gc815-73 | Serotype O:5,27 | (80) |
| 8081 | Serotype O:8 | (81) |
| <i>Escherichia coli</i> | | |
| BL21 Star TM (DE3) PLysS | | Invitrogen |
| DH10B | | |
| Plasmids | | |
| pTM100 | | (82) |
| pTM100_OmpF | Complementation plasmid with wild type <i>ompF</i> gene | This work |

| | | |
|-----------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| | cloned into pTM100 | |
| pSW25T | Suicide vector | (83) |
| pSW25T_OmpF | Complementation suicide plasmid with wild type <i>ompF</i> gene cloned into pSW25T | This work |
| pCDF Duet-1 | pCloDF13 replicon, T7lac promoter and 2 MCS sites each with an optional N-terminal His ₆ tag sequence. Streptomycin resistance marker. | Novagen |
| pET21a(+) | ColE1 (pBR322) replicon, T7lac promoter, N-terminal T7 tag sequence and optional C-terminal His ₆ Tag sequence. Ampicillin resistance marker. | Novagen |
| pCDF Duet-1 Gp37 | Phage TG1 <i>ORF250</i> (4-1,830 bp) cloned in frame into MCS1 of pCDF Duet-1 for expression of N-terminal His ₆ tagged protein Gp37. | This study |
| pCDF Duet-1 Gp37-Gp38 | Phage TG1 <i>ORF251</i> cloned into MCS2 of pCDF Duet-1 Gp37 for co-expression of N-terminal His ₆ tagged Gp37 and tail fiber assembly chaperone Gp38. | This study |
| pET21a(+) Gp57A | Phage TG1 <i>ORF143</i> cloned into MCS of pET21a(+) for expression of general trimerization chaperone Gp57A. | This study |
| <hr/> | | |
| Bacteriophages | | |
| TG1 | | This study |
| φR1-RT | | This study |
| <hr/> | | |

Table 2. Lytic activities of phages TG1 and ϕ R1-RT. The sensitivity was tested on 160 *Yersinia* species strains (Table S1) at 25°C.

| <i>Yersinia</i> species | Phage sensitive serotypes ^a | Serotypes with phage sensitive (S) and resistant (R) strains | Phage resistant serotypes ^b |
|------------------------------|-------------------------------------------------------------------------------|--------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <i>Y. enterocolitica</i> | O:1[2], O:2 [2], O:3 [16], O:5 [9], O5,27 [10], O:6 [2], O:7,8 [2], O:9 [13], | O:6,30 [1S/2R], O:6,31 [1S/1R] | O:1,2,3 [1], O:4 [1], O:4,32 [1], O:8 [14], O:10 [4], O:13 [1], O:13a,13b [1], O:13,7 [2], O13,18 [1], O:14 [1], O:20 [2], O:21 [3], O:25 [1], O:25,26,44 [1], O:26,44 [1], O28,50 [1], O:34 [1], O:35,36 [1], O35,52 [1], O:41(27),K1 [1], O41(27),42 [1], O:41(27),42,K1 [1], O:41,43 [1], O:41(27),43 [2], O:50 [1], K1 NT[2], NT[3] |
| <i>Y. aleksiciae</i> | | | O:16 [2] |
| <i>Y. aldovae</i> | | | UT [2] |
| <i>Y. bercovieri</i> | | | O:58,16 [2], NT [1], UT[2] |
| <i>Y. frederiksenii</i> | | | O:3 [1], O:16 [1], O:35 [1], O:48 [1], K1 NT[1], NT[1], UT[2] |
| <i>Y. intermedia</i> | | | O16,21 [1], O:52,54 [1], UT[2] |
| <i>Y. kristensenii</i> | | | O:3 [1], O:12,25 [1], NT[2], UT[3] |
| <i>Y. mollareti</i> | | | O:3 [1], O:59(20,36,7) [1], UT[2] |
| <i>Y. nurmii</i> | | | UT[1] |
| <i>Y. pekkanenii</i> | | | UT[1] |
| <i>Y. pseudotuberculosis</i> | | | I [2], O:1b [2], O:3 [2] |
| <i>Y. rohdei</i> | | | UT[2] |
| <i>Y. ruckeri</i> | | | NT[1], UT[5] |

^aThe number of strains studied is given in brackets. Phage ϕ R1-RT sensitivity was tested only with the 109 UH-source strains (Table S1).

^bNT, non-typeable and either cross-reacting or not agglutinating with *Y. enterocolitica* O:3, O:5, O:8 or O:9 antisera. UT, untyped.

FIGURE LEGENDS

Figure 1. Bacteriophage ϕ R1-RT and TG1 morphology by electron microscopy. **Panel A.** ϕ R1-RT virions at 39,440x magnification. The virion head and tail are indicated, as well as long tail fibers (LTF) and a baseplate with protruding tail pins (B). Scale bar, 100 nm. **Panel B.** A ϕ R1-RT virion at 84,320x magnification. A baseplate with protruding tail pins (B), and a neck and collar with neck fibers (A) can be observed. Scale bar, 50 nm. **Panel C.** A ϕ R1-RT virion at 108,800x magnification. Suggested long tail fibers (LTF) can be seen bent up towards the head along the tail sheath, as described for bacteriophage T4 (84). Scale bar, 50 nm. **Panel D.** Bacteriophage TG1 virion shown at 150,000x magnification. A neck and collar with neck fibers (A), a baseplate with protruding tail pins (B), and an extended long tail fiber (LTF) can be observed. Scale bar indicates size in nm.

Figure 2. ProgressiveMauve alignment of phage TG1 and ϕ R1-RT. The genome of ϕ R1-RT [HE956709] is indicated on the top and that of TG1 [KP202158] is shown in the bottom of the figure. The degree of sequence similarity between regions is given by a similarity plot within the coloured blocks with the height of the plot proportional to the average nucleotide identity. Below these are illustrated the phage genes as outlined boxes on the plus (above horizontal) and minus (below horizontal) strands.

Figure 3. Phylogenetic analysis of the large terminase subunit protein sequences of phages TG1, ϕ R1-RT and related bacteriophages. The phylogenetic analysis was constructed using “one click” at phylogeny.fr using MUSCLE for multiple alignment and PhyML for phylogeny (85).

Figure 4. Phage ϕ R1-RT does not propagate at 37°C. **Panel A.** The growth curves of *Y. enterocolitica* infected with ϕ R1-RT. Bacteria were cultured with different concentrations of phage particles in LB at 4°C, 10°C, 16°C, 22°C, and 37°C. Each graph represents the average of five replicates. Note the different scales used for the X-axis at different temperatures. **Panel B.** Analysis of the *ompF* gene expression (left) and protein abundance (right) at different temperatures. The mean expression levels of the *ompF* gene were obtained from RNA-sequencing analysis. The production levels of the OmpF protein was obtained from normalized mean spectral values for the proteins detected by the LC-MS/MS analysis. Error bars represent the calculated standard deviation.

Figure 5. Phages ϕ R1-RT and TG1 use OmpF and LPS inner core heptose region of *Y. enterocolitica* O:3 as receptors. **Panel A.** Adsorption experiments were performed with different LPS and *ompF* mutants, with the complemented strains, and with the pseudolysogen. All strains are OmpF positive with the exception of YeO3-c-R1-Cat17. The TG1 and ϕ R1-RT adsorptions to the bacteria at 5 min are shown as residual PFU percentages. Error bars indicate standard deviations. The no bacteria control (LB) and strains used for adsorptions are indicated below the columns. The LPS chemotypes of the strains are indicated on top of the columns. **Panel B.** The schematic structures of the *Y. enterocolitica* O:3 LPS molecules of different chemotypes (86). Please note that *Y. enterocolitica* O:3 carries simultaneously the S and Ra type LPS molecules. This is indicated in panel A by a plus sign. O-ag, O-antigen or O polysaccharide; OC, outer core hexasaccharide; IC, inner core; LA, lipid A.

Figure 6. Confocal immunofluorescence microscopy images of *Y. enterocolitica* cells after incubation with LTF protein Gp37 derived from phage TG1. Gp37 decorates the cell surface of *Y. enterocolitica* strain K14 of serotype O:9 (a), *Y. enterocolitica* strain gc815-73 of serotype O:5,27 (c), and *Y. enterocolitica* strain 6471/76 of serotype O:3 (e) grown at 25°C, whereas *Y. enterocolitica* strain 8081 of serotype O:8 (g) does not show cell decoration with Gp37. Similar

images to that presented in g were observed when the same strains were grown at 37°C. Differential interference contrast microscopy images of a, c, e and g, are shown in b, d, f and h, respectively. Scale bar represent size in μm .

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Plasmid constructs for chaperone-assisted expression of the distal long tail fiber protein Gp37 of phage TG1. The location of the origins of replication, antibiotic resistance genes (Sm R, streptomycin resistance; Amp R, ampicillin resistance), relevant promoters (T7 lac promoter), Lac I repressor, Multiple cloning sites (yellow) and sequences coding phage Gp37 (red), and chaperones Gp38 and Gp57A (green) are presented.

Figure S2. Map of the phage $\phi\text{R1-RT}$ genome [HE956709]. The genes are shown by different-colored arrows. The arrow direction indicates the coding direction of the genes. The genes encoding putative proteins with an assigned function are shown in black (see also Table S3). The locations of tRNA encoding genes are shown in blue. Hypothetical proteins with an unknown function are depicted in yellow. Homing endonuclease genes are shown in orange. Putative host promoters are shown as pink triangles above the sequence, and putative rho independent terminators are shown as green triangles below the sequence.

Figure S3. Map of the phage TG1 genome [KP202158]. The genes are shown by different-colored arrows. The arrow direction indicates the coding direction of the genes. The genes encoding putative proteins with an assigned function are shown in black (see also Table S2). The locations of tRNA encoding genes are shown in blue. Hypothetical proteins with an unknown function are depicted in yellow. Homing endonuclease genes are shown in orange. Putative host promoters are shown as pink triangles above the sequence, and putative rho independent terminators are shown as green triangles below the sequence.

Figure S4. Multiple sequence alignment of the long tail fiber (Gp37) sequences of phage TG1 and $\phi\text{R1-RT}$. Multiple sequence alignment was performed using Clustal W via Geneious R9 software version 9.0.2. (Biomatters Ltd). Positions which have a single, fully conserved aa residue (100% similarity) are highlighted in black; aa present in 2 of the sequences are highlighted in grey. The homologous phage T4 Gp37 sequence was included in the alignment or comparison.

Figure S5. Multiple sequence alignment of the short tail fiber (Gp12) protein sequences of phage TG1 and $\phi\text{R1-RT}$. Multiple sequence alignment was performed using Clustal W via Geneious R9 software version 9.0.2. (Biomatters Ltd). Positions which have a single, fully conserved aa residue (100% similarity) are highlighted in black; aa present in 2 of the sequences are highlighted in grey. The homologous phage T4 Gp12 sequence was included in the sequence alignment for comparison.

Figure S6. Protein mass fingerprinting of phage TG1 Gp37. The amino acid sequence of phage TG1 Gp37 is shown. Peptide fragments from the analysis of gel slices corresponding to the reduced form of the protein and identified via protein mass fingerprinting are underlined and shown in bold.

Figure S7. Whole genome phylogeny of phages TG1, $\phi\text{R1-RT}$ and related bacteriophages. The phylogenetic tree was generated using using “one click” at phylogeny.fr using MUSCLE for multiple alignment and PhyML for phylogeny (85).

Figure S8. Expression of N-terminal His₆ tagged phage TG1 Gp37. 4-15% Tris-HCl SDS-PAGE run at 4°C, 100V. Lane 1, pCDF-Duet-1 Gp37 (unheated sample); Lane 2, pCDF-Duet-1 Gp37 under reduced conditions (sample heated at 100°C for 10 min in the presence of SDS and β-mercaptoethanol); Lane 3, pCDF-Duet-1 Gp37-Gp38 (unheated sample); Lane 4, pCDF-Duet-1 Gp37-Gp38 reduced conditions; Lane 5, pCDF-Duet-1 Gp37-Gp38 co-expressed with pET21a(+) Gp57A (unheated sample); Lane 6 pCDF-Duet-1 Gp37-Gp38 co-expressed with pET21a(+) Gp57A reduced conditions; Lane 7, pCDF-Duet-1 Gp37 co-expressed with pET21a(+) Gp57A (unheated sample); Lane 8, pCDF-Duet-1 Ggp37 co-expressed with pET21a(+) Gp57A reduced conditions; Lane M, molecular weight markers.

Figure S9. CLUSTAL W multiple sequence alignment of the OmpF proteins of *Y. enterocolitica* and related species. The external loops, indicated by brown highlighting and box, were identified based on OmpF alignment of YeO3 OmpF with that of *E. coli*, shown at the bottom. The N-terminal signal-peptide is indicated by blue highlighting and box. Use the zoom-in option to see details of the alignment.

SUPPLEMENTARY TABLES

Table S1. List of *Yersinia* strains used in phage host range experiments

Table S2. Primers used in this work

Table S3. Bacterial strains used to examine the cross infectivity of phage TG1

Table S4. Annotations of bacteriophage TG1 and φR1-RT genes

Table S5. Putative Phage TG1 promoters

Table S6. Putative Phage φR1-RT promoters

Table S7. Predicted terminator sequences of phage TG1

Table S8. Predicted terminator sequences of phage φR1-RT

Table S9. TG1 genome BLASTN analysis

Table S10. Whole genome sequencing statistics after de novo assembly of φR1-RT resistant mutants.

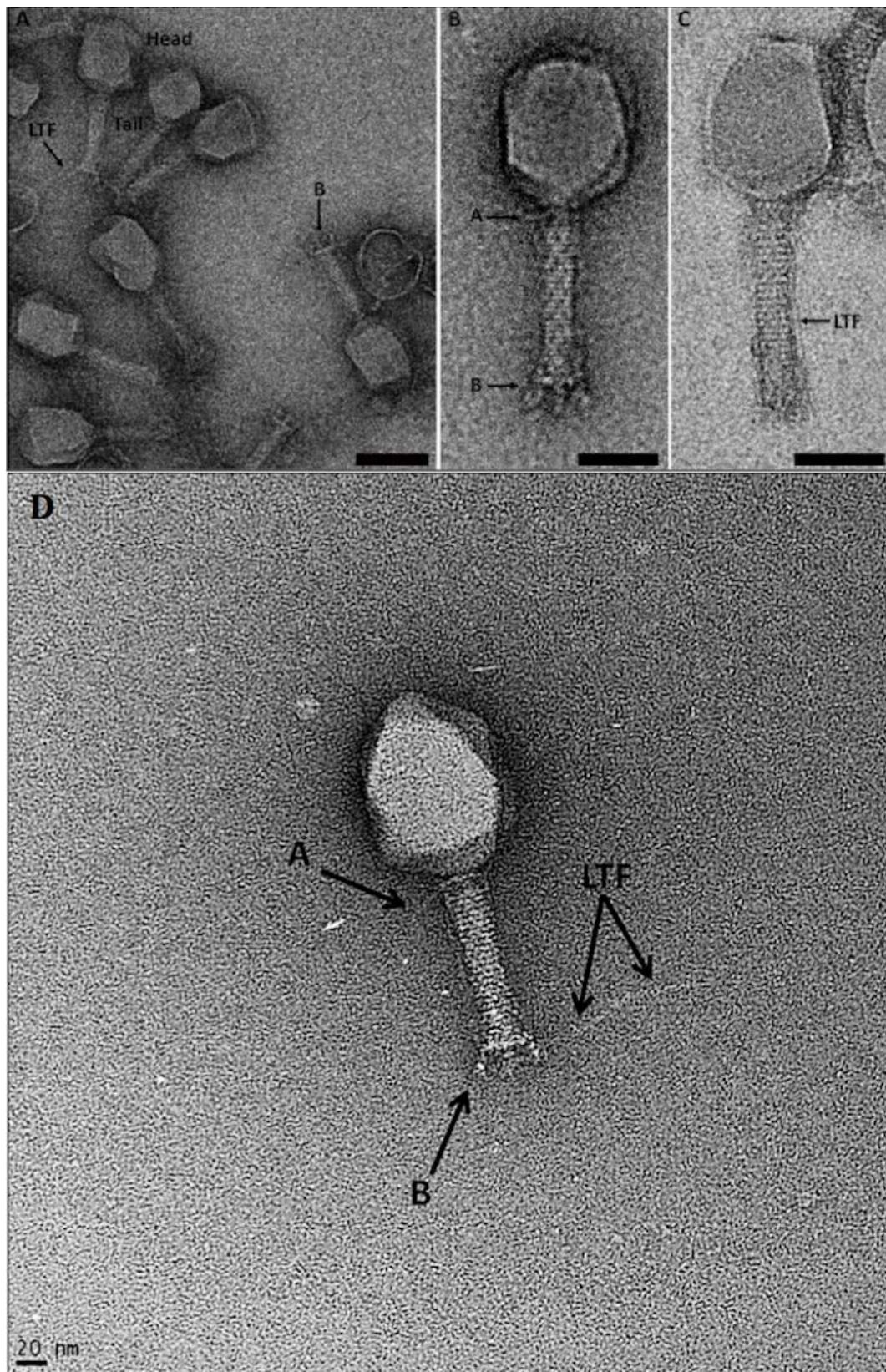


Figure 1.

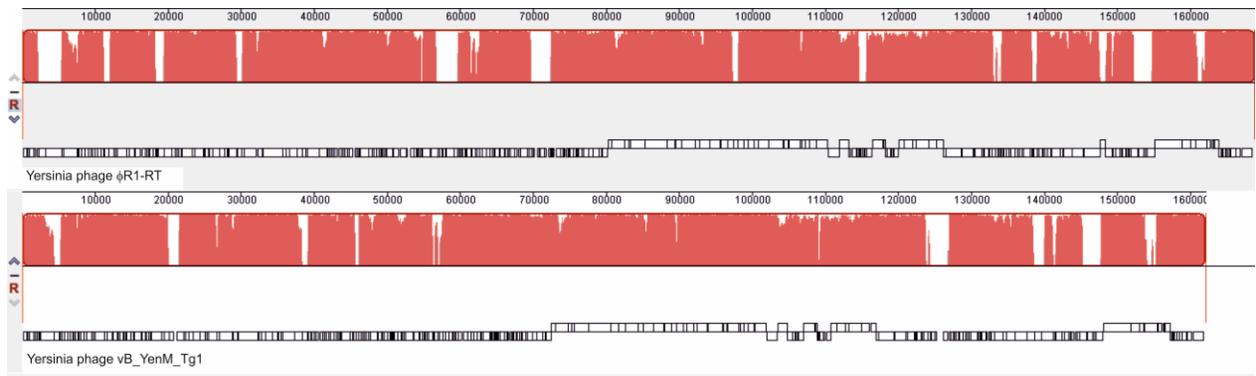


Figure 2.

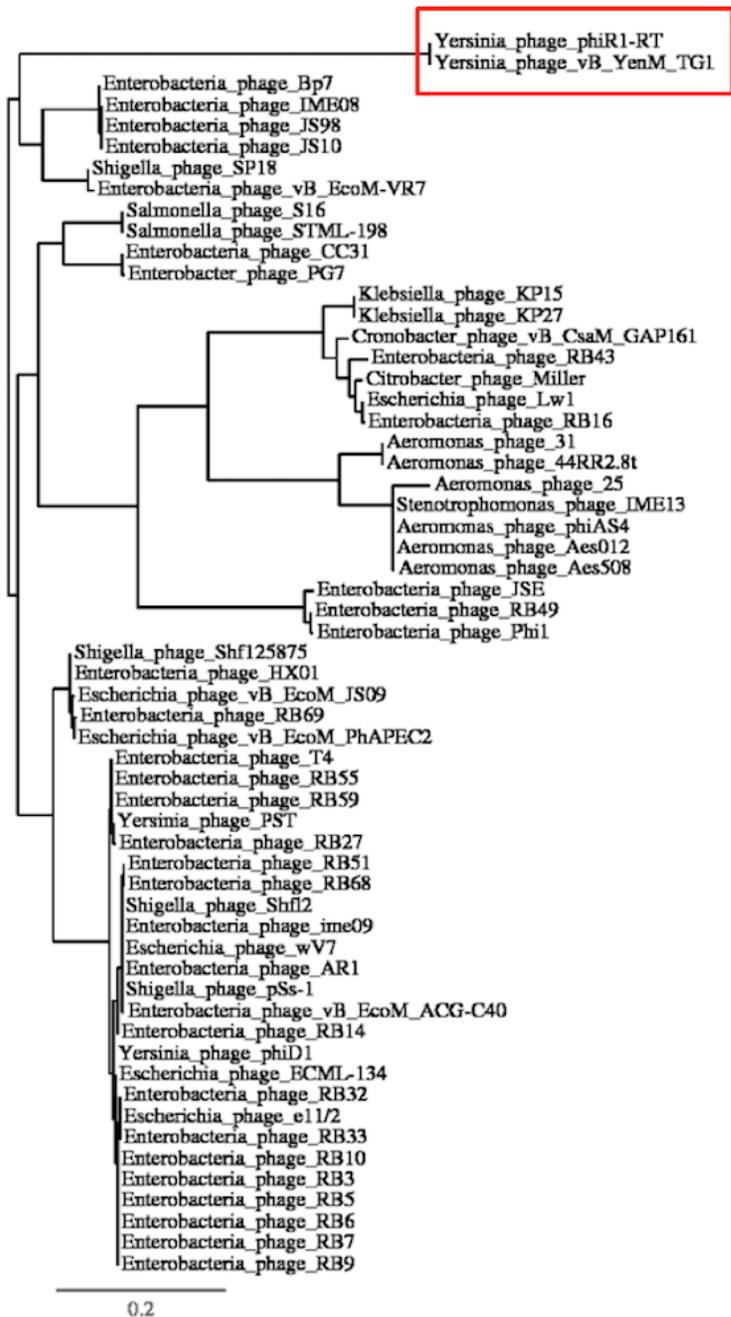


Figure 3

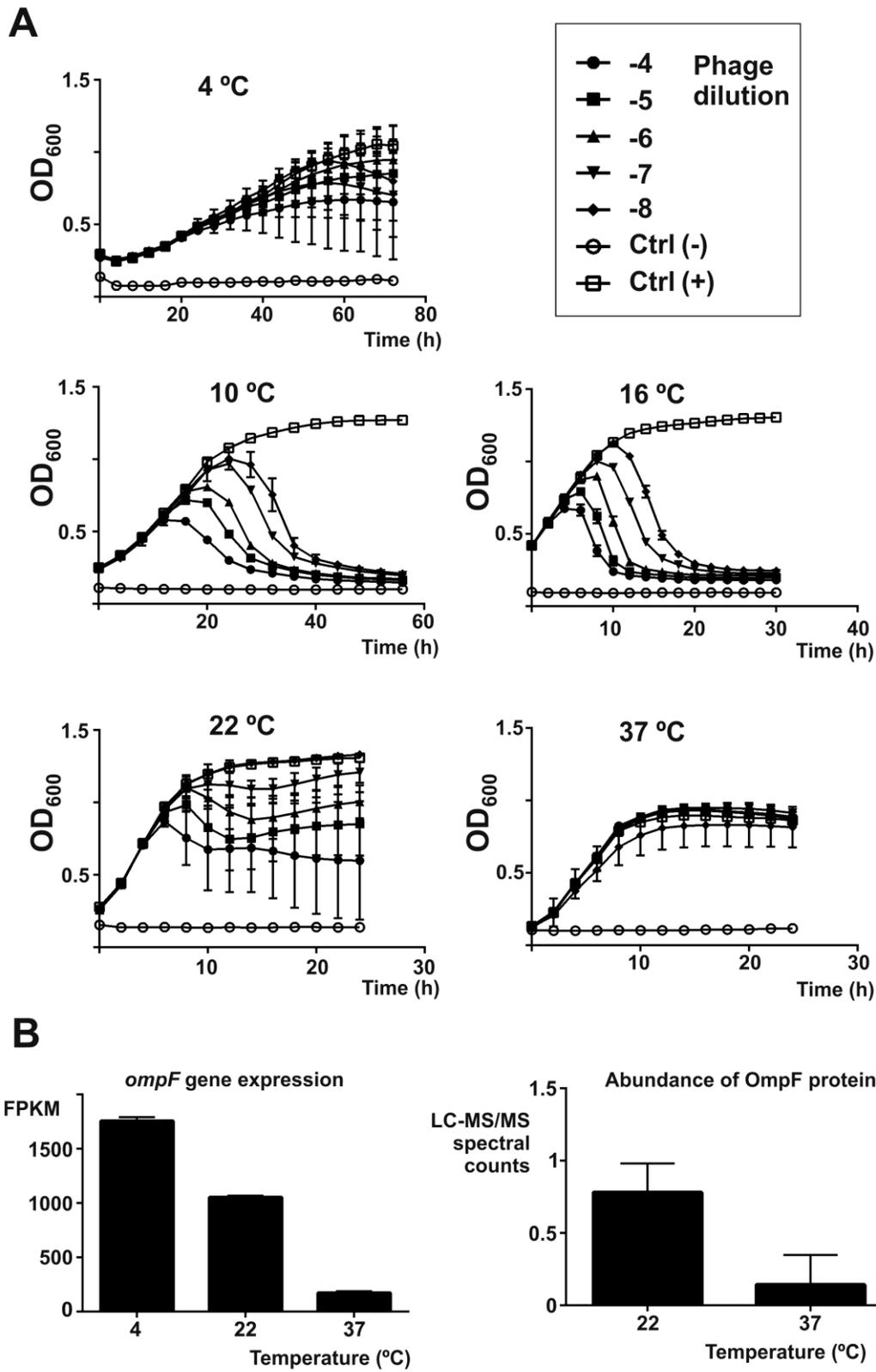


Figure 4

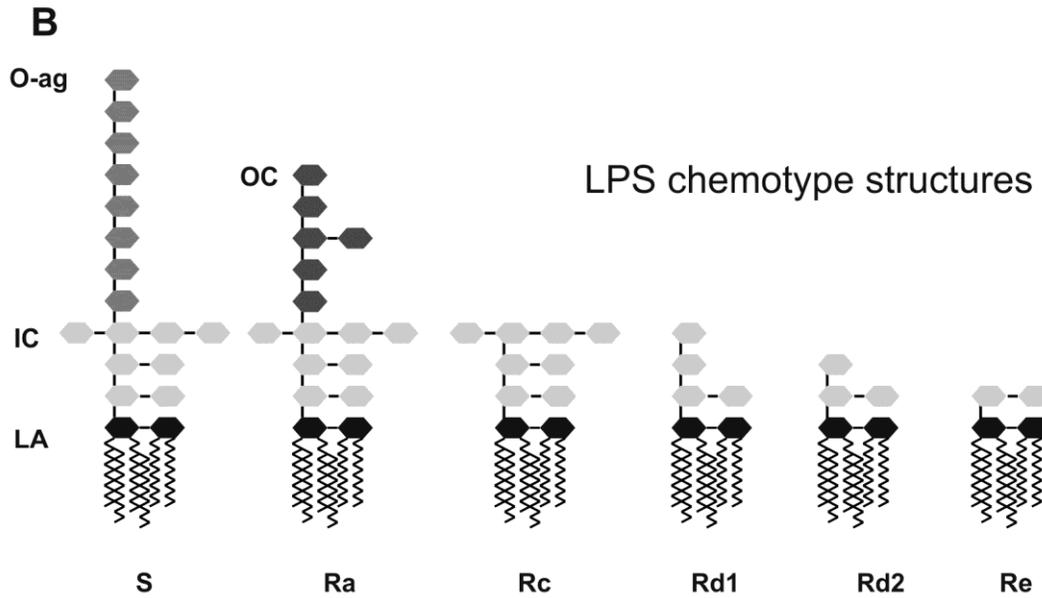
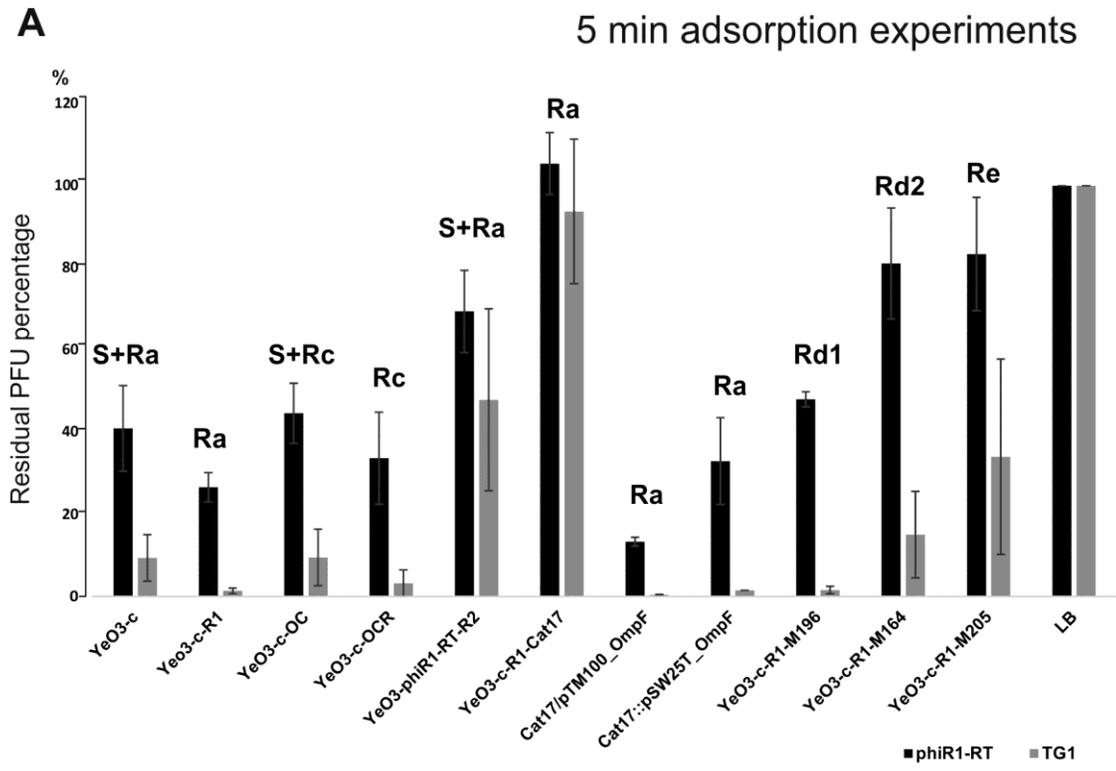


Figure 5

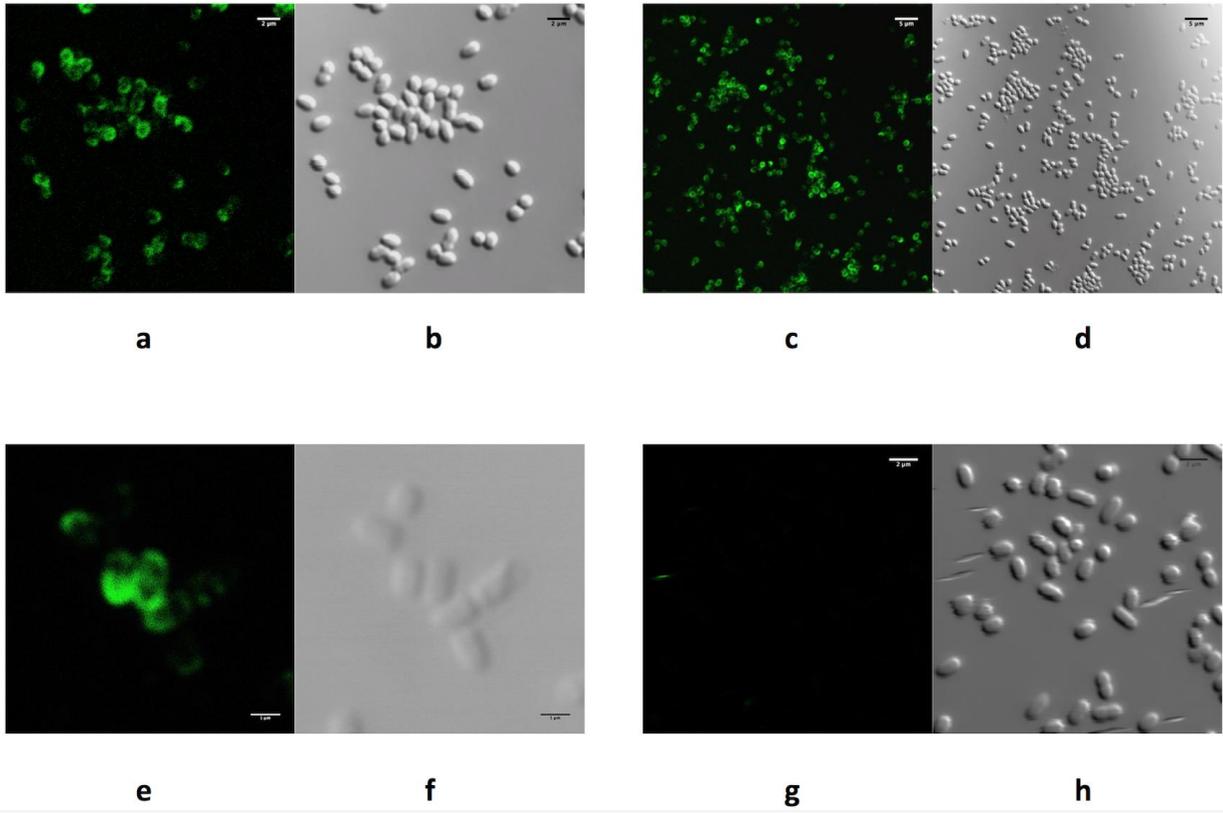


Figure 6